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14. ABSTRACT Treatment of patients with advanced stages of breast cancer remains an unresolved clinical problem. The main objectives of this study are to determine whether immunotherapy sensitizes tumor to chemotherapy and to identify some of the main mechanisms of this effect. We investigated the possibility of a direct synergy between immunotherapy and chemotherapy <i>in vitro</i> . We found that pre-treatment of tumor target cells with doxorubicin or paclitaxel significantly increased cytotoxic effect of T-lymphocytes. Importantly, that effect was antigen-specific, since it was observed only in tumor cells loaded with specific but not a control peptide. In contrast, pre-treatment of splenocytes did not result in enhancement of target cell killing. In parallel experiments we have determined that both drugs increased the expression of p53 in tumor cells. However, that increase observed only after 48 hr of treatment and therefore could not contribute to observed sensitization of tumor cells to CTLs. To determine the effect of the combined treatment <i>in vivo</i> , mammary carcinoma TUBO was established s.c. in BALB/c mice. Dendritic cell vaccine alone slowed down tumor growth, which was consistent with previous results obtained by many laboratories. Paclitaxel had similar effect. However, in both cases tumor growth resumed in about a week after end of the treatment. In a sharp contrast, tumor size was substantially reduced in mice treated with combination of DC vaccine and chemotherapy. Most of the mice rejected tumor. Thus, this data indicates that a direct combination of chemotherapy with cancer vaccine provides substantial antitumor effect via sensitization of tumor cells to CTLs. These experimental models will be used for investigation the mechanisms of this phenomenon.					
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Introduction

Treatment of locally advanced or metastatic breast cancer remains a very difficult clinical problem. Chemotherapy is the treatment of choice for most of those patients. It is given as an adjuvant or neo-adjuvant settings alone or in combination with hormone therapy or Herceptin. Two classes of the drugs are primarily used: anthracyclines (doxorubicin, epirubicin, mitoxantrone) and taxanes (paclitaxel, docetaxel). In addition to the well-described toxicity the efficacy of the treatment remains relatively low. Median survival for patients with metastatic breast cancer is 18-24 months. Among patients treated with systemic chemotherapy 16.6% achieved complete responses and only 3.1% remained in complete remission for more than 5 years (1). Patients with locally advanced breast cancer (LABC) have a poor prognosis when treated with surgery and radiotherapy. Preoperative (neo-adjuvant) chemotherapy has been developed as an alternative therapeutic strategy as it allows surgical intervention in patients who present with bulky primary disease. In general, neo-adjuvant chemotherapy results in a complete-response rate of 10% to 35%. However, the five-year overall survival rate is only 5% to 20% (2). All these results compel the development of new approaches to therapy of breast cancer. Immunotherapy of breast cancer has not yet delivered tangible clinical results. Although some clinical trials performed in recent years demonstrated encouraging results, most of the trials showed rather limited clinical response(3). It appears that tumor escape mechanisms prevent effective recognition and elimination of tumors. New approaches are necessary to make cancer immunotherapy clinically effective. One of the most attractive approaches to cancer therapy is a combined modality treatment. However, the well-known immunosuppressive effect of chemotherapy has established a widely accepted notion that the direct combination of chemotherapy and immunotherapy will be ineffective due to the negative effect of chemotherapy on the immune system. Recently we and others have reported findings from clinical trials, which may challenge that paradigm. Patients with advanced stages of different types of cancer were treated with different vaccines. Direct clinical effect of those vaccines was quite limited. However, patients showed high objective clinical response rate to chemotherapy that immediately followed immunotherapy (4-8). Taken together these recent data suggest a possibility of new paradigm in cancer treatment. Immunotherapy can substantially enhance the effect of chemotherapy. It could be especially important for patients with advanced stage breast cancer. These data suggest a new paradigm that vaccination may be most effective in direct combination with chemotherapy. The main objectives of this study are to determine whether immunotherapy sensitizes tumor to chemotherapy and to identify some of the main mechanisms of this effect.

Body

Experiments *in vitro*. In preliminary experiments we have identified optimal dose of chemotherapeutic drugs. The challenge was to select the dose that would be eventually effective but would not kill tumor cells within first 24-48 hrs to provide sufficient time window to observe potential effect of CTLs. We determined such doses that would induce tumor cell death only after 72 hr of treatment. At the same time those doses did not affect tumor cell viability within first 24 hr. All experiments *in vitro* were performed within first 24 hr after start of treatment.

We investigated the possibility of a direct synergy between immunotherapy and chemotherapy *in vitro*. For initial experiments we used well-developed model of cytotoxic activity mediated by CTLs that utilizes EL-4 target cells loaded with control or specific peptides. Tumor-free C57BL/6 mice were immunized twice with dendritic cells (DC) activated with LPS and loaded with H2-K^b bound p53-derived peptide (KYMCSNSSCM). Splenocytes were isolated, re-stimulated with the specific peptide and then used as effectors in CTL assay against EL-4 target cells loaded with either specific (p53) or control H2-K^b bound peptide (RAHYNIVTF). Either splenocytes or EL-4 cells were pretreated overnight with 1.5 mg/ml doxorubicin (DOX), 12.5 nM paclitaxel (TAX). After overnight incubation cells were washed and used in 6-hr CTL assay. Pre-treatment of target cells with DOX or TAX significantly increased cytotoxicity. Importantly, that effect was antigen-specific, since it was observed only in EL-4 cells loaded with specific but not a control peptide. In contrast, pre-treatment of splenocytes did not result in enhancement of target cell killing. Moreover, the level of cytotoxicity was slightly decreased. These experiments were performed 4 times now and all demonstrated consistent results. Then we asked what effect these drugs would have if CTLs and tumor cells will be treated at the same time. CTLs and tumor cells were pre-treated with TAX or DOX separately overnight and then cultured together in 6-hr CTL assay. The level of cytotoxicity in treated cells was significantly ($p < 0.05$) higher than in non-treated cells. This data indicates TAX and DOX have strong effect on tumor cells that overcome some negative effect on CTL activity. This would be consistent with previously reported results of clinical trials. We could conclude that chemotherapy sensitize tumor cells to the effect of CTLs.

In parallel experiments we tried to evaluate possible mechanisms of this effect. First, we tested the possibility that TAX and DOX could up-regulate p53 expression in tumor cells. EL-4 tumor cells were treated with these drugs for different time, whole cell lysate was prepared, and the level of p53 was evaluated by Western blotting. TAX and DOX both indeed increased the expression of p53 in tumor cells. However, that increase observed only after 48 hr of treatment and therefore could not contribute to observed sensitization of tumor cells to CTLs. To address this issue further we repeat CTL assay described above using OT-1 transgenic T-cells as effectors. These cells recognize chicken ovalbumin (OVA)-derived epitope SIINFEKL. As targets we used EL-4 cells loaded with control or specific peptide (SIINFEKL). As in case of p53 peptide DOX and TAX sensitized tumor cells to killing by CTLs. These experiments demonstrated that observed effect of chemotherapy is not restricted to only p53 and most likely represent general phenomenon.

Next we evaluated possible role of Fas-FasL in the effect of chemotherapy. TAX and DOX increased expression of FASL on the surface of T cells. This was measured by flow cytometry using specific antibody. These findings are consistent with previously published observations. To test the possibility that FAS-FASL interaction plays critical role in chemotherapy mediated sensitization of tumor cells to CTLs we used goat anti-mouse FAS ligand neutralizing antibody (R&D Systems). CTLs and tumor cells were pre-treated with 50 µg/ml of this antibody for 1 hr and then cultured together in the presence of the antibody. As a control we used 50 µg/ml goat IgG. Anti-FasL antibody did not prevent the ability of DOX and TAX to enhance killing of tumor cells by CTLs. These experiments were performed only once and require confirmation. If they are confirmed this would indicate that the observed effect of chemotherapy was not mediated by FAS-FASL.

Experiments *in vivo*. In preliminary experiments we established proof-of-concept using EL-4 tumor-bearing mice and immunization with p53 peptide loaded dendritic cells (DC). We have observed that combination of TAX and immunotherapy dramatically reduced tumor size. Based on these experiments we have designed experiments using breast tumor model.

To determine the effect of the combined treatment *in vivo*, mammary carcinoma TUBO was established s.c. in BALB/c mice. This tumor express Her2/neu antigen. Five days after tumor injection mice were split into four groups: 1 – control, untreated mice; 2 – mice treated with activated dendritic cells (DC) loaded with Her2/neu-derived peptide; 3 – mice treated with TAX alone and 4 – mice treated with combination of DC vaccine and TAX. Each group included 5 mice. DCs (5×10^5 cells) were administered s.c. three times with 7-day interval. TAX was injected i.p. 3 days after second DC vaccination and again 3 days after third DC vaccination. DC

vaccine alone slowed down tumor growth, which was consistent with previous results obtained by many laboratories. TAX had similar effect. However, in both cases tumor growth resumed in about a week after end of the treatment. In a sharp contrast, tumor size was substantially reduced in mice treated with combination of DC vaccine and TAX. Two mice in group 4 have rejected tumors, whereas no mice in groups 1,2,and 3 have done that. Tumor size in remaining mice from group 4 was significantly smaller than in mice from other groups. At the end of the experiment (5 weeks after tumor injection) tumor size in group 4 was more than three fold lower than in groups 2 and 3 and more than 5 times lower than in group 1 ($p < 0.05$ in two-sided Mann-Whitney test). Currently we are repeating these experiments to confirm these findings.

Key Research Accomplishments

- In experimental model of breast cancer we have determined that immunotherapy has synergized with chemotherapy in potent antitumor activity
- In experiments *in vitro* we have found that chemotherapy sensitize tumor cells to the effect of CTLs. At the same time, pre-treatment of CTLs with chemotherapeutic drugs did not improve cytotoxicity. Thus, synergistic effect of immunotherapy and chemotherapy was mediated primarily by the effect of chemotherapy on tumor cells.
- This effect was not mediated by up-regulation of p53 expression or FasL expression

Reportable Outcomes

None at this moment

Conclusions

The data obtained so far indicates that a direct combination of chemotherapy with cancer vaccine provides substantial antitumor effect via sensitization of tumor cells to CTLs. These experimental models will be used for further investigation the mechanisms of this important phenomenon.

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